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Jin Montclare

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Polytechnic University - Brooklyn
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Polytechnic Institute of New York University
Brooklyn, NY 11201 -3840

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14. ABSTRACT
Inspired by nature's approach to biomaterials synthesis, we employ a bottom up assembly of proteins to fabricate defined fibers that are robust and stable. We have decorated these protein fibers with bioorthogonal chemical moieties in the form of unnatural amino acids to achieve stability in fiber assembly and defined inorganic•protein complexes with magnetic properties. The resulting set of proposed biopolymers bear tremendous impact in understanding the principles for designing highly robust protein fibers in general. Moreover, the coupling of inorganic materials with biomaterials provides a means for patterning magnetite crystals on assemblies

15. SUBJECT TERMS
coiled-coils, protein fibers, biomaterials, nanoparticles, self-assembly, protein engineering

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a. REPORT	b. ABSTRACT	c. THIS PAGE			Jin Montclare
UL	UL	UL	UL		19b. TELEPHONE NUMBER 718-260-3679

Report Title

Final Report: Bottom-up Assembly of Engineered Protein Fibers

ABSTRACT

Inspired by nature's approach to biomaterials synthesis, we employ a bottom up assembly of proteins to fabricate defined fibers that are robust and stable. We have decorated these protein fibers with bioorthogonal chemical moieties in the form of unnatural amino acids to achieve stability in fiber assembly and defined inorganic•protein complexes with magnetic properties. The resulting set of proposed biopolymers bear tremendous impact in understanding the principles for designing highly robust protein fibers in general. Moreover, the coupling of inorganic materials with biomaterials provides a means for patterning magnetite crystals on assemblies.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Received		Paper
08/05/2012	1.00	Susheel Gunasekar, Luona Anjia, Hiroshi Matsui, Jin K Montclare. Effects of Divalent Metals on Nanoscopic Fiber Formation and Small Molecule Recognition of Helical Proteins, Advanced Functional materials , (02 2012): 0. doi:
08/17/2014	4.00	Jasmin Hume, Jennifer Sun, Rudy Jacquet, P. Douglas Renfrew, Jesse A. Martin, Richard Bonneau, Malcolm Lane Gilchrist, Jin Kim Montclare. Engineered Coiled-Coil Protein Microfibers, Biomacromolecules, (06 2014): 0. doi: 10.1021/bm5004948
08/17/2014	5.00	Haresh T. More, Joseph A. Frezzo, Jisen Dai, Seiichi Yamano, Jin K. Montclare. Gene delivery from supercharged coiled-coil protein and cationic lipid hybrid complex, Biomaterials, (08 2014): 7188. doi: 10.1016/j.biomaterials.2014.05.005
TOTAL:	**3**	

Number of Papers published in peer-reviewed journals:

(b) Papers published in non-peer-reviewed journals (N/A for none)

Received	Paper
TOTAL:	

Number of Papers published in non peer-reviewed journals:

(c) Presentations

Number of Presentations: 0.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received	Paper

TOTAL:

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received	Paper

TOTAL:

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):

(d) Manuscripts

Received	Paper	
02/15/2015	8.00	Jasmin Hume, Raymond Chen, Rudy Jacquet, Michael Yang, Jin Montclare. Tunable Conformation-Dependent Engineered Protein•Gold Nanoparticle Nanocomposites , Biomacromolecules (01 2015)
02/15/2015	7.00	Haresh More, Kevin Zhang, Nikita Srivastiva, Joseph Frezzo, Jin Montclare. Stimuli Responsive Fluorinated Protein Engineered Coiled-coil Fibers, Biomacromolecules (01 2015)

TOTAL: **2**

Number of Manuscripts:

Books

Received		Book
08/15/2013	3.00	Haresh More, Ching-Yao Yang, Jin Montclare. Posttranslational Modification of Proteins Incorporating Nonnatural Amino Acids, Germany: Wiley-VCH Verlag GmbH & Co. KGaA., (03 2013)

TOTAL: **1**

Received	Book Chapter

TOTAL:

Patents Submitted

Protein Engineered Systems for Dual Small Molecule and Gene Delivery.
Protein Nanofibers.

Patents Awarded

Awards

2014 Distinguished Award for Excellence, Dedication to Invention, Innovation and Entrepreneurship
2014 Executive Leadership in Academic Technology and Engineering Fellow
2013 NYAS Chemical Biology Steering Committee
2013 NSF I-Corps Award

Graduate Students

NAME	PERCENT_SUPPORTED	Discipline
Haresh More	1.00	
Jasmin Hume	1.00	
Joseph Frezzo	0.10	
Rudy Jacquet	0.00	
FTE Equivalent:	**2.10**	
Total Number:	**4**	

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Faculty Supported

NAME	PERCENT_SUPPORTED	National Academy Member
Jin Montclare	0.25	
FTE Equivalent:	**0.25**	
Total Number:	**1**	

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED	Discipline
Kevin Zhang	0.10	Biomolecular Science
Michael Yang	0.10	Chemistry
Raymond Chen	0.20	Chemical and Biomolecular Engineering
Jennifer Sun	0.10	Biomolecular Science
FTE Equivalent:	**0.50**	
Total Number:	**4**	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period:	4.00
The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:.....	4.00
The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:.....	1.00
Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):.....	3.00
Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:.....	0.00
The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense	0.00
The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields:	0.00

Names of Personnel receiving masters degrees

NAME	
Joseph Frezzo	
Rudy Jacquet	
Total Number:	**2**

Names of personnel receiving PHDs

NAME	
Haresh More	
Jasmin Hume	
Total Number:	**2**

Names of other research staff

NAME	PERCENT_SUPPORTED

FTE Equivalent:
Total Number:

Sub Contractors (DD882)

Inventions (DD882)

5 **Protein Engineered Systems for Dual Small Molecule and Gene Delivery.**

Patent Filed in US? (5d-1) Y
Patent Filed in Foreign Countries? (5d-2) N
Was the assignment forwarded to the contracting officer? (5e) N
Foreign Countries of application (5g-2):

5a: Jin Montclare

5f-1a: NYU Polytechnic School of Engineering

5f-c: 6 Metrotech Center

Brooklyn NY 11201

5a: Haresh More

5f-1a: NYU Polytechnic School of Engineering

5f-c: 6 Metrotech Center

Brooklyn NY 11201

5 **Protein Nanofibers**

Patent Filed in US? (5d-1) Y
Patent Filed in Foreign Countries? (5d-2) N
Was the assignment forwarded to the contracting officer? (5e) N
Foreign Countries of application (5g-2):

5a: Jin Montclare

5f-1a: NYU Polytechnic School of Engineering

5f-c: 6 Metrotech Center

Brooklyn NY 11201

5a: Jasmin Hume

5f-1a: NYU Polytechnic School of Engineering

5f-c: 6 Metrotech Center

Brooklyn NY 11201

Scientific Progress

Technology Transfer

The gene delivery work has led to the initiation of a startup company tentatively named Brooklyn Biologics.

Abstract:
Inspired by nature's approach to biomaterials synthesis, we employ a bottom up assembly of proteins to fabricate defined fibers that are robust and stable. We have decorated these protein fibers with bioorthogonal chemical moieties in the form of unnatural amino acids to achieve stability in fiber assembly and defined inorganic•protein complexes with magnetic properties. The resulting set of proposed biopolymers bear tremendous impact in understanding the principles for designing highly robust protein fibers in general. Moreover, the coupling of inorganic materials with biomaterials provides a means for patterning magnetite crystals on assemblies.

Statement of the problem studied:
In this grant period, we explored the design and synthesis of coiled-coil protein fibers such that we are able to control the assembly from the nano- to mesoscale. We have been able to improve stability and protein fiber assembly while also template inorganic metal nanoparticle to create new organic•inorganic hybrids. While studying these systems, we also developed supercharged coiled-coils mixed with cationic lipids for applications in gene delivery.

Summary of the most important results:
Summaries are provided in discrete projects as part of the whole grant. For those projects in which manuscripts are published or under review, abstracts along with figures are provided. For the project that is not completed, a more lengthy description is provided.

1) Engineered Coiled-Coil MicroFiber [1]
The fabrication of de novo proteins able to self-assemble on the nano- to meso-length scales is critical in the development of protein-based biomaterials in nanotechnology and medicine. Here we report the design and characterization of a protein engineered coiled-coil that not only assembles into microfibers, but also can bind hydrophobic small molecules. Under ambient conditions and a pH 4, the protein forms fibers with nanoscale structure possessing large aspect ratios formed by bundles of α-helical homopentameric assemblies, which further assemble into mesoscale fibers in the presence of curcumin through aggregation. Surprisingly, these biosynthesized fibers are able to form in conditions of remarkably low concentrations. Unlike previously designed coiled-coil fibers, these engineered protein microfibers can bind the small molecule curcumin throughout the assembly, serving as a depot for encapsulation and delivery of other chemical agents within protein-based 3D microenvironments.

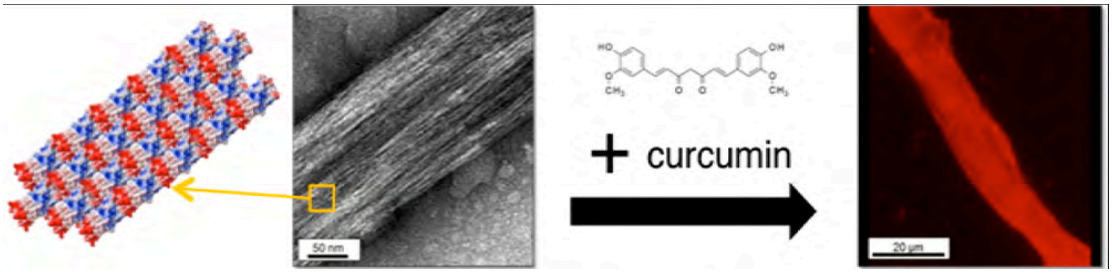


Figure 1. Transmission electron micrograph of Q fiber, 10 μM, pH 4. Schematic representation of Q fiber assembly with staggered positive (red) and negative (blue) regions of the pentamer. Confocal microscopy fluorescence images of Q protein fiber in the presence of 50 μM curcumin at pH 4, 50 mM PB at room temperature, illustrating size change from nanofiber to microfiber.

2) Stimuli Responsive Fluorinated Protein Engineered Coiled-Coil Fibers [2]

We design and characterize fluorinated coiled-coil proteins able to assemble into robust nano- and micro-fibers. Fluorination is achieved biosynthetically by residue-specific incorporation of 5,5,5-trifluoroleucine (TFL). The fluorinated proteins C+TFL and Q+TFL are highly α-helical as confirmed via circular dichroism (CD) and more resistant to thermal denaturation compared to their non-fluorinated counterparts, C and Q. The fluorinated proteins demonstrate enhanced fiber assembly with higher order structure. Ionic strength dependent fiber assembly is observed for fluorinated as well as wild type proteins with robust fiber formation 100 mM NaCl. The proteins reveal metal ion dependent small molecule recognition and supramolecular assemblies. In the presence of Zn (II), enhanced thermal stability and fiber assembly is observed for the fluorinated proteins and their non-fluorinated counterparts. Whereas Ni (II) promotes aggregation with no fiber assembly, the stabilization of α-helix by Zn (II) results in enhanced binding to curcumin to by the fluorinated proteins. Surprisingly, the non-fluorinated proteins exhibit multiple fold increase in curcumin binding in the presence of Zn (II). In the context of growing number of protein-based fiber assembly, these fluorinated coiled-coil proteins introduce a new paradigm in the development of highly stable self-assembling fibers that promotes the binding and release of small molecules in response of external cues.

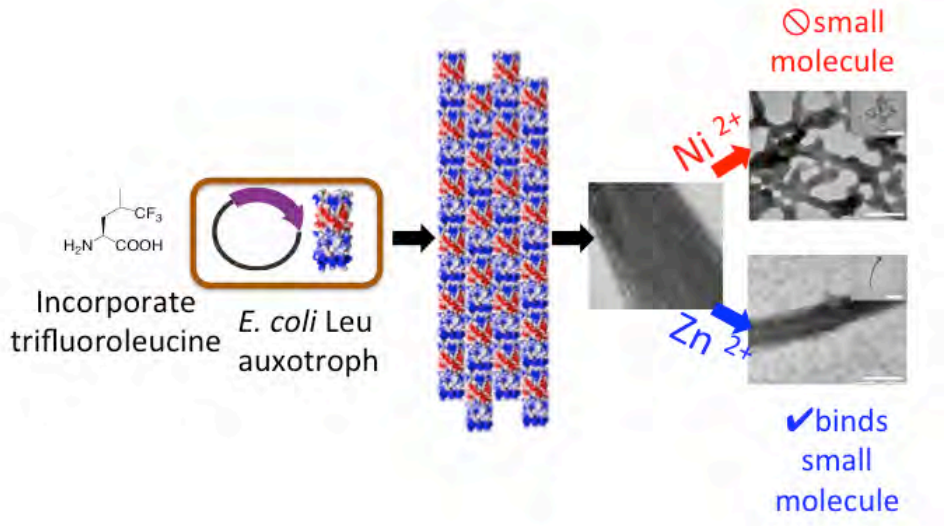


Figure 2. Incorporation of TFL into C and Q proteins lead to improved stability and robust assembly under ambient, neutral conditions. The C+TFL and Q+TFL proteins exhibit Ni (II) and Zn (II)-dependent assembly. In the presence of Ni (II), aggregation is induced, whereas in the presence of Zn (II) fiber assembly and stability is enhanced, leading to improved binding to curcumin.

3) Tunable Conformation-Dependent Engineered Protein•Gold Nanoparticle Nanocomposites [3]

We demonstrate the fabrication of protein•gold nanoparticle (AuNP) nanocomposites *in situ*, leading to distinct assemblies dependent upon protein secondary structure. In the presence of pentameric coiled-coil proteins C and Q, which contain histidine tags and helical content, templation of AuNP results in precipitation of the protein•AuNP composites with AuNPs that are 6.5 nm in diameter, creating macromolecular assemblies on the micrometer scale. In the absence of the histidine tags the resulting Cx and Qx proteins, which exhibit lower helicities, stabilize soluble protein•AuNP composites AuNPs that are 4.5 nm in diameter for several days without aggregating. By manipulating protein structure via external triggers, such as TFE, we obtain control over the macromolecular conformation and overall physicochemical properties. These hybrid protein•AuNP assemblies can be readily deposited on electrodes, where they can serve as a tunable bio-nanocomposite kinetic barrier.

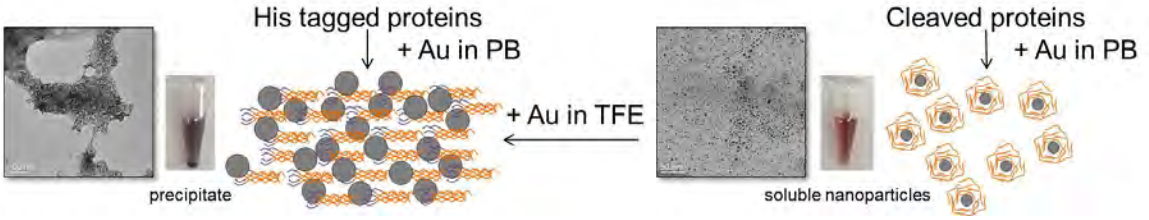


Figure 3. Protein nanoparticle film formation results from protein self-assembly, dictated by structure. Templation of gold nanoparticles on structured proteins leads to film formation via aggregation, while unstructured proteins solvate nanoparticles. In the presence of TFE, structure is induced in cleaved proteins allowing solvated nanoparticles to aggregate. Electrochemical properties indicate that these materials can be suitable for biosensor development.

4) Magnetite Templation on Functionalized Protein Biomaterials

Coiled-coil proteins based on COMPcc have been used as a building block in the creation of functional materials capable of templating magnetic nanoparticles. Unnatural amino acid incorporation of an azide-bearing residue, L-azidohomoalanine (AHA), into proteins C and Q, whose sequences are based on COMPcc, offered a chemical handle upon which an orthogonal magnetite templating peptide, CMms6, was attached. Alkyne-functionalized CMms6 was attached to the AHA-bearing proteins through a copper catalyzed click chemistry reaction and monitored molecular weight shifts in SDS-PAGE gel electrophoresis.

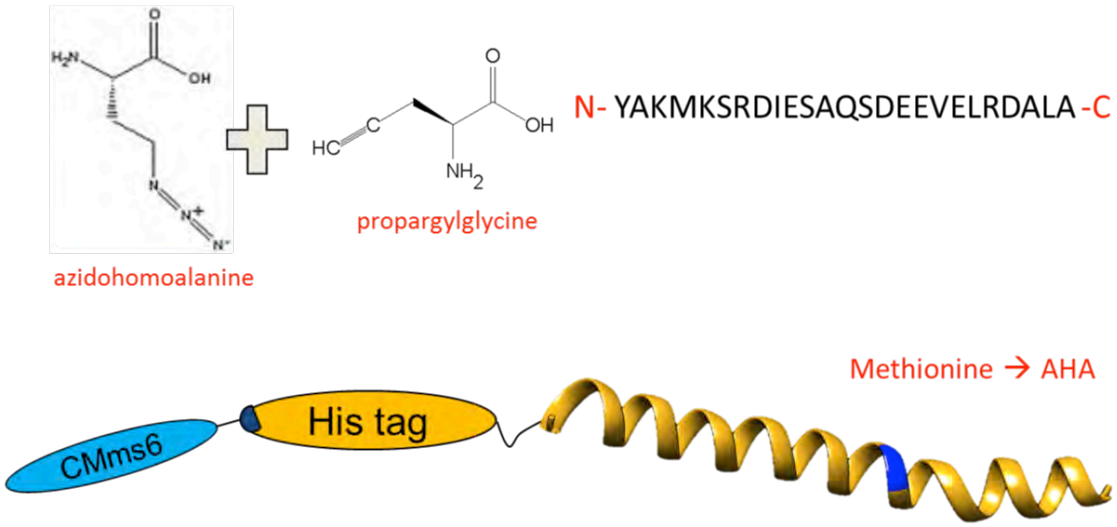


Figure 4. Schematic overview of functionalization of CAHA and QAHA with an alkyne-functionalized CMms6 via click chemistry.

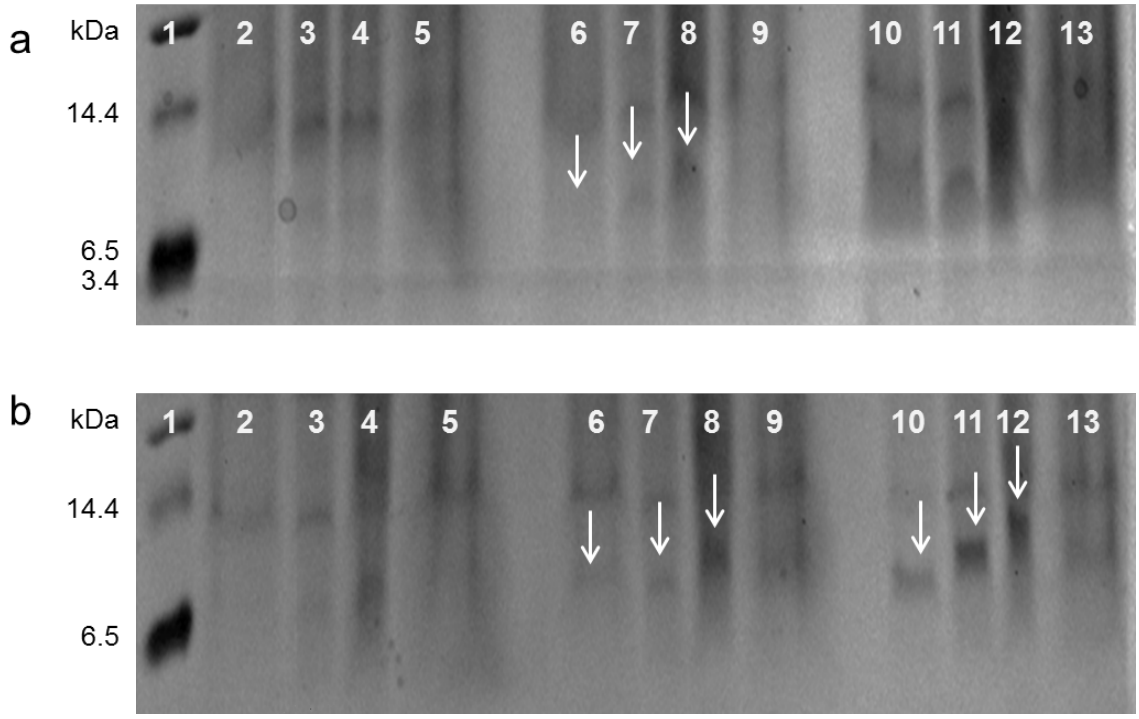


Figure 5. SDS-PAGE of click chemistry as a function of time, with sodium ascorbate and varying concentrations of prg-CMms6. (a) C+AHA lysate. 1. Ladder, 2-5: No prg-CMms6, 6-9: 200 µM prg-CMms6, 10-13: 500 µM prg-CMms6, where lanes 2, 6, and 10 were taken at the beginning of the reaction; 3, 7, and 11 represent 20 h of incubation; 4, 8, and 12 represent 28 h of incubation; and 5, 9, and 13 represent 48 h of incubation. (b) Q+AHA lysate. 1. Ladder, 2-5: No prg-CMms6, 6-9: 200 µM prg-CMms6, 10-13: 500 µM prg-CMms6, where lanes 2, 6, and 10 were taken at the beginning of the reaction; 3, 7, and 11 represent 20 h of incubation; 4, 8, and 12 represent 28 h of incubation; and 5, 9, and 13 represent 48 h of incubation.

Magnetic nanoparticles (MNPs) were synthesized through coprecipitation of $FeCl_3$ and $FeSO_4$ and reduced with NaOH to produce octahedral nanoparticles approximately 8.6 nm in diameter in the absence of CMms6 and diameters ranging from 16.3 to 12.2 nm in the presence of CMms6 concentrations ranging from 200 μM to 1 mM, respectively. Transmission electron microscopy confirmed that regular, octahedral MNPs were synthesized in the presence of CMms6, whereas MNPs crystallized with only AHA protein resulted in MNPs of irregular morphology, including needle-shaped crystals.

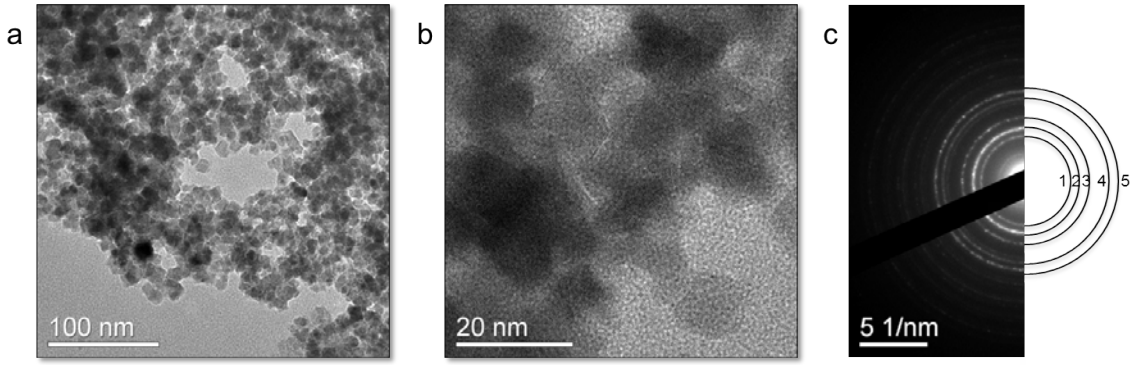


Figure 5. Transmission electron micrographs for MNPs formed in the presence of 1 mM prg-CMms6 via coprecipitation in 50 mM PB, pH 8 (a, b). Electron diffraction pattern of MNPs formed in the presence of prg-CMms6 (c) displayed rings that were used to calculate **d** -spacing of the crystals. Scale bars are 100 nm
(a), 20 nm (b), and 5 nm-1 (c).

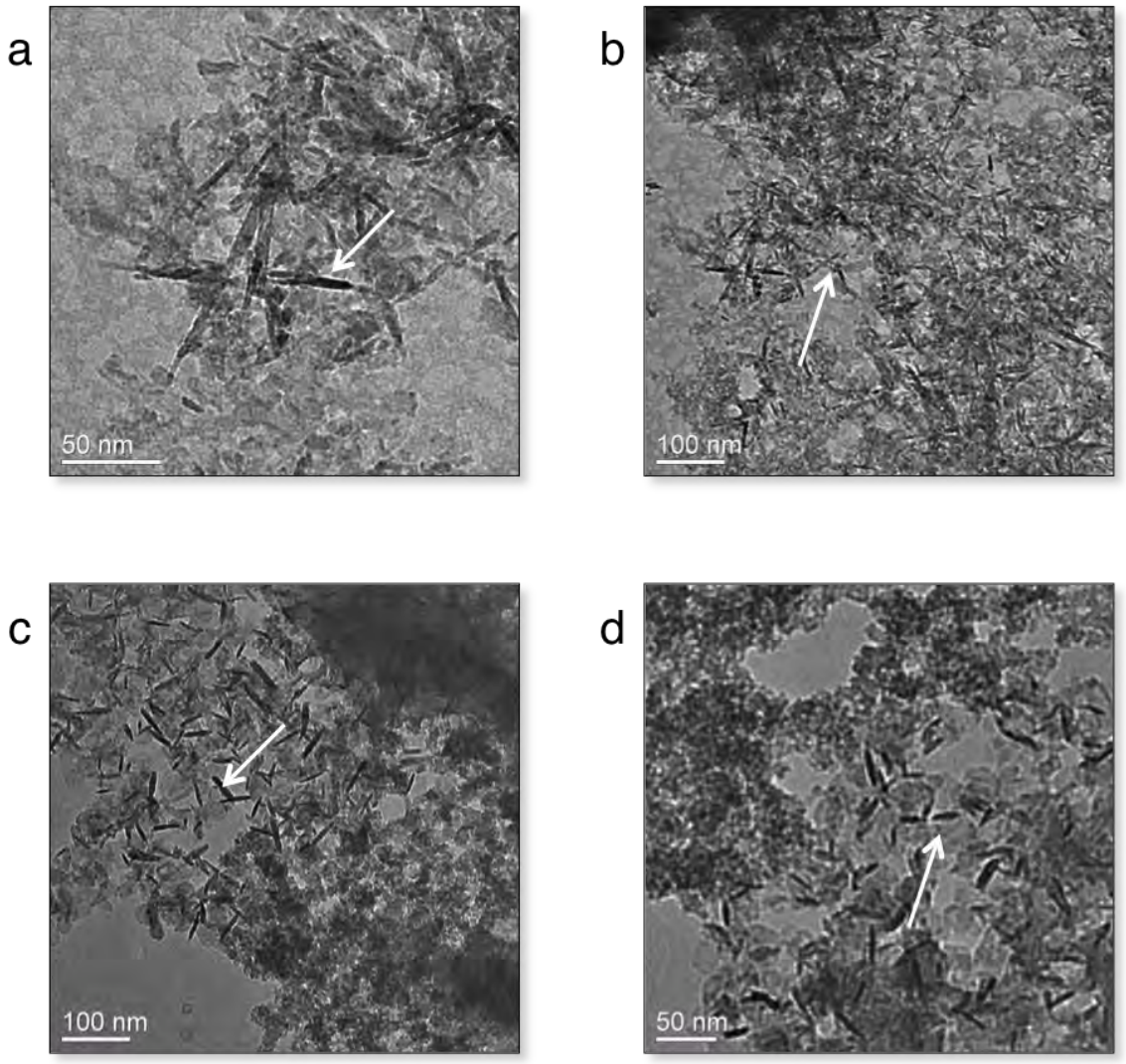


Figure 6. TEM of unclicked C+AHA + MNP (a, b) and unclicked Q+AHA + MNP (c, d). Concentration of C+AHA was 23 **μ** M and Q+AHA was 11 **μ** M, with molar ratios of iron salts and reducing agent normalized accordingly. Presence of needle-like crystals is indicated by white arrows. Scale bars represent 100 nm in (b) and (c) and 50 nm in (a) and (d). Samples imaged without uranyl acetate stain.

Whole cell lysate containing AHA protein clicked with CMms6 was used for templation of MNPs and studied with TEM. Crystallization and stabilization of MNPs by proteins is important in biomedical fields where magnetic materials can be made biocompatible or ligand-targeted with proteins in areas such as magnetic resonance imaging as well as targeted drug delivery through use of magnetic fields.

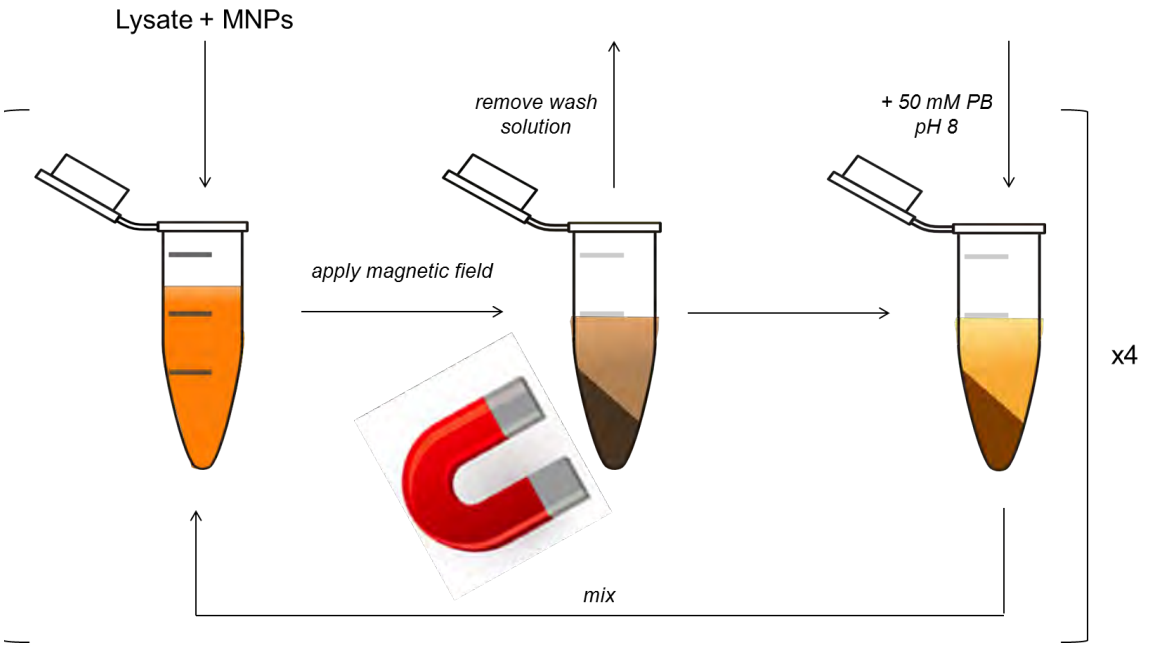


Figure 7. Schematic of setup for magnetic separation experiments.

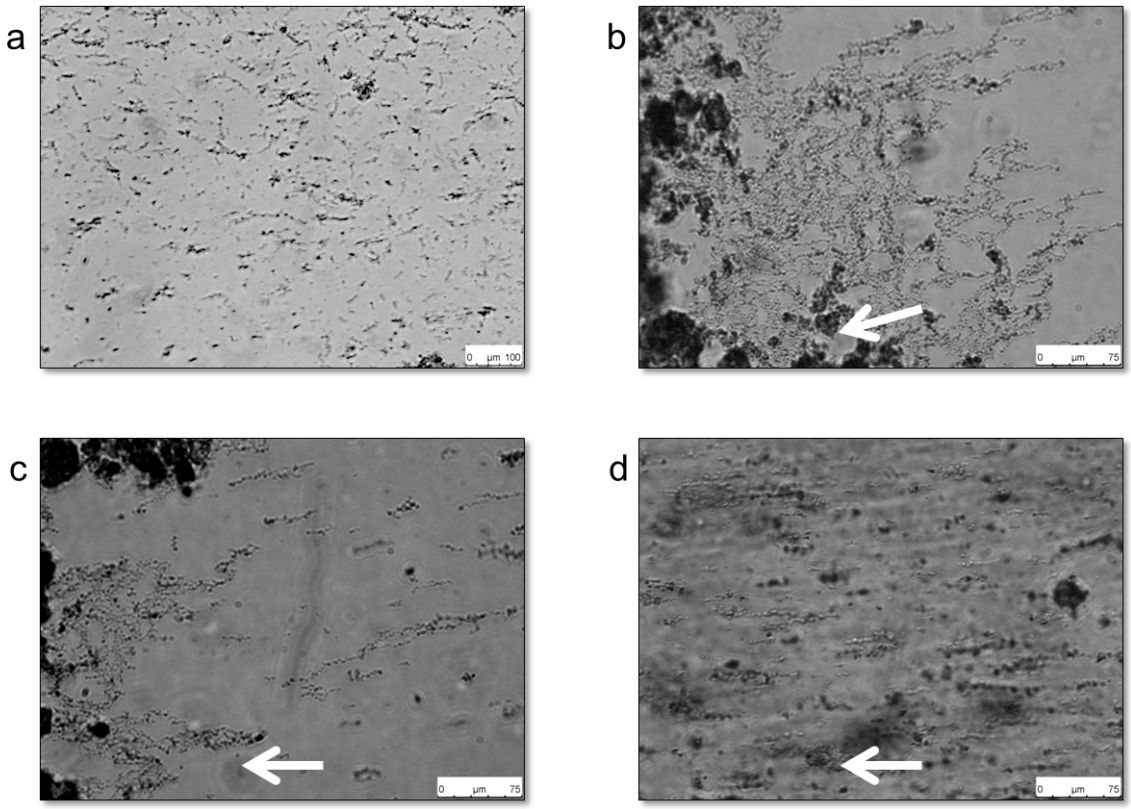
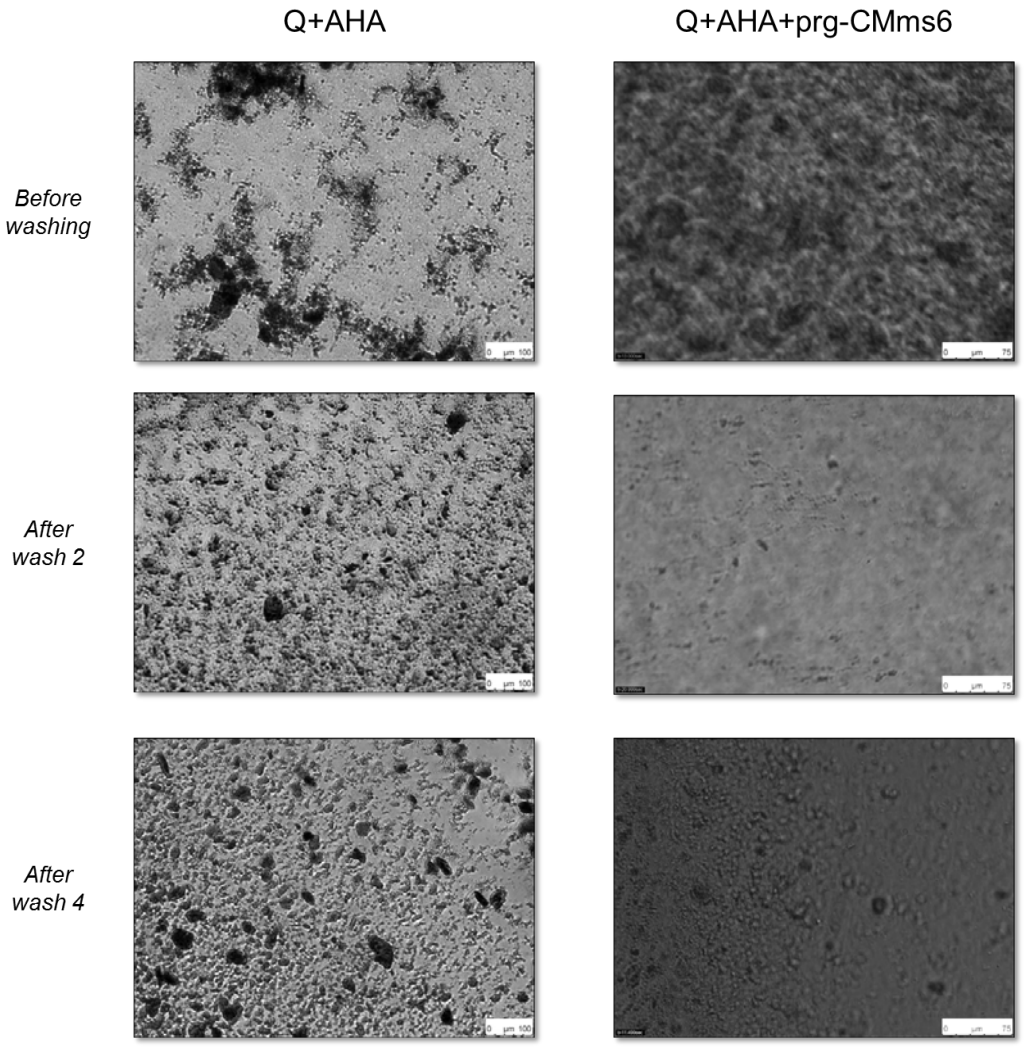


Figure 8. Phase contrast microscopy of MNPs synthesized in the presence of 200 µM prg-CMms6 in 50mM PB pH 8 before (a) and after (b, c, d) the application of a magnetic field. Direction of the magnetic field is indicated by the white arrows. Scale bar represents 100 µm in (a) and 75 µm in (b), (c), and (d).



(6)

Figure 9. Phase contrast microscopy of MNPs synthesized in 50 mM PB pH 8 incubated with Q+AHA lysate and Q+AHA+prg-CMms6 lysate before (top) and after 2 washes (middle) and 4 washes (bottom) via magnetic separation. Scale bar represents 100 μm in top left, middle left, and bottom left images and 75 μm in top right, middle right, and bottom right images.

The manipulation of these solutions with a neodymium magnet demonstrated that prg-CMms6 and Q+AHA+prg-CMms6 samples were retarded in traveling through the solution compared to MNPs in Q+AHA samples. This suggests that the presence of CMms6 influences the magnetite assembly and organization.

5) Gene delivery from supercharged coiled-coil protein and cationic lipid hybrid complex [4]

A lipoproteoplex comprised of an engineered supercharged coiled-coil protein (CSP) bearing multiple arginines and the cationic lipid formulation FuGENE HD (FG) was developed for effective condensation and delivery of nucleic acids. The CSP was able to maintain helical structure and self-assembly properties while exhibiting binding to plasmid DNA. The ternary CSP•DNA(8:1)•FG lipoproteoplex complex demonstrated enhanced transfection of β-galactosidase DNA into MC3T3-E1 mouse preosteoblasts.

The lipoproteoplexes showed significant increases in transfection efficiency when compared to conventional FG and an mTat•FG lipopolyplex with a 6- and 2.5-fold increase in transfection, respectively. The CSP•DNA(8:1)•FG lipoproteoplex assembled into spherical particles with a net positive surface charge, enabling efficient gene delivery. These results support the application of lipoproteoplexes with protein engineered CSP for non-viral gene delivery.

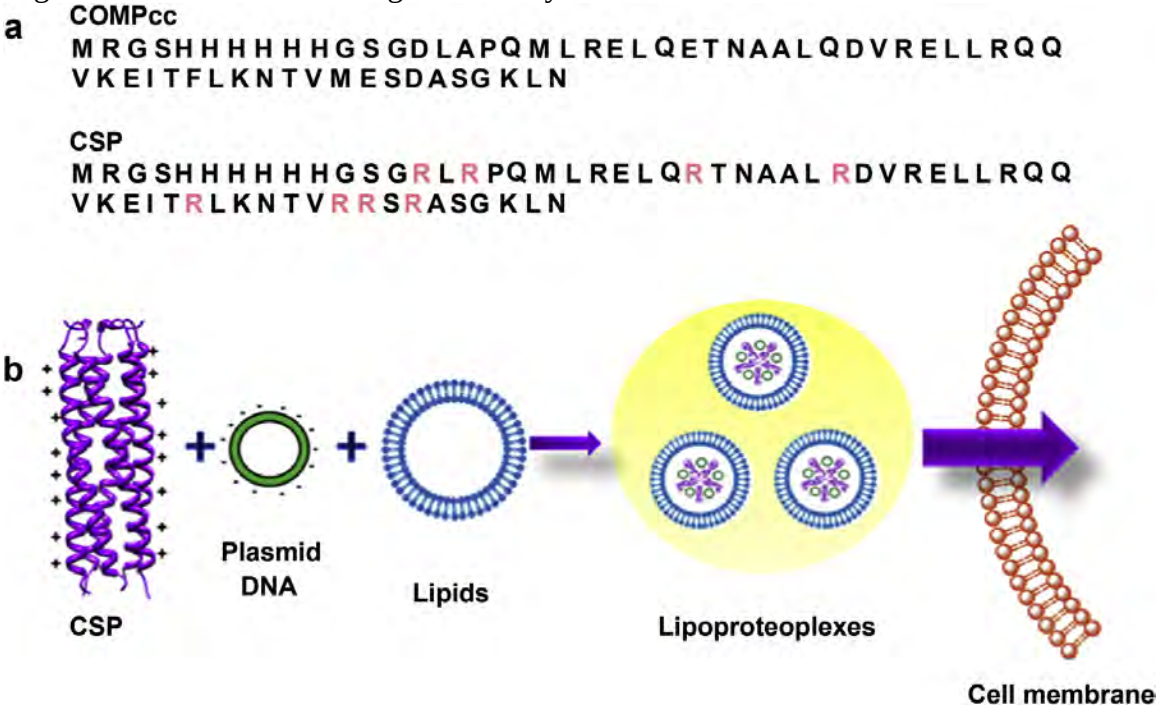


Figure 10. a) Aligned sequences of COMPcc and CSP with mutated arginine residue positions shown in red. b) Schematic of CSP complexation with plasmid DNA and a ternary complex with cationic lipids to form lipoproteoplexes for gene delivery.

Bibliography:

1. Hume, J., J. Sun, R. Jacquet, P.D. Renfrew, J.A. Martin, R. Bonneau, M.L. Gilchrist, and J.K. Montclare, *Engineered Coiled-Coil Protein Microfibers.* Biomacromolecules, 2014. **15**(10): p. 3503-3510.
2. More, H.T., K.S. Zhang, N. Srivastiva, J.A. Frezzo, and J.K. Montclare, *Stimuli Responsive Fluorinated Protein Engineered Coiled-coil Fibers.* Biomactormolecues, 2015. **submitted**.
3. Hume, J., R. Chen, R. Jacquet, M. Yang, and J.K. Montclare, *Tunable Conformation-Dependent Engineered Protein•Gold Nanoparticle Nanocomposites* Biomacromolecules, 2015. **submitted**.
4. More, H.T., J.A. Frezzo, J. Dai, S. Yamano, and J.K. Montclare, *Efficient Gene Delivery from Supercharged Coiled-coil Protein and Cationic Lipid Nanocomplexes.* . Biomaterials, 2014. **35**: p. 7188-7193.